

Figure 1. Effects of carvedilol on coronary hemodynamics in ischemic myocardium. A and B show CPP and CBF, respectively. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

Measurement of Infarct Size and Collateral Blood Flow

In protocol 4, the area of myocardial necrosis and the area at risk¹⁶ were measured in all of the dogs upon completion of the protocol by an operator who had no knowledge of the treatment given to each animal. Infarct size was expressed as a percentage of the area at risk.

Regional myocardial blood flow was determined as described previously.¹⁷ Nonradioactive microspheres (Sekisui Plastic Co) made of inert plastic were labeled with bromine. Microspheres were administered at 80 minutes after the start of coronary occlusion. The radio fluorescence of the stable heavy elements was measured with a wavelength dispersive spectrometer (PW 1480, Phillips Co). Because the level of energy emitted is characteristic of specific elements, it was possible to quantify the radio fluorescence of the heavy element with which the microspheres were labeled. Myocardial blood flow was calculated according to the following formula: $\text{time flow} = (\text{tissue count}) \times (\text{reference flow}) / (\text{reference count})$, and was expressed in milliliters per minute per gram wet weight. Endomyocardial blood flow was measured at the inner half of the left ventricular wall.

Exclusion Criteria

To ensure that all of the animals used for analysis of infarct size in protocol 4 were healthy and were exposed to a similar extent of ischemia, the following standards were used for exclusion of unsatisfactory dogs: (1) subendocardial collateral blood flow $>15 \text{ mL} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$, (2) a heart rate $>170 \text{ bpm}$, and (3) >2 consecutive attempts required to terminate ventricular fibrillation using low-energy DC pulses applied directly to the heart.

Statistical Analysis

Statistical analysis was performed by use of ANOVA^{18,19} to compare data among the groups. When ANOVA indicated a significant difference, paired data were compared by use of the Bonferroni test. Changes of the hemodynamic and metabolic parameters over time were assessed by ANOVA with repeated measures. Results were expressed as the mean \pm SEM, with a value of $P < 0.05$ being considered significant.

Results

Effects of Carvedilol on VAD(Ado) in Nonischemic Myocardium

Neither systemic hemodynamic parameters (mean blood pressure, 101.0 ± 2.1 versus $98.6 \pm 3.2 \text{ mm Hg}$ and heart rate, 130.2 ± 3.7 versus $128.0 \pm 3.3 \text{ bpm}$) nor FS ($20.1 \pm 1.0\%$ versus $21.5 \pm 1.0\%$) changed during the infusion of carvedilol.

In contrast, CBF was increased (98.4 ± 8.5 versus $112.6 \pm 9.6 \text{ mL} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$, $P < 0.05$), as was VAD(Ado) (40.9 ± 4.0 versus $68.6 \pm 5.5 \text{ nmol/L}$, $P < 0.05$).

Effects of Either Carvedilol or Propranolol on VAD(Ado) During Coronary Hypoperfusion

Administration of either 8-SPT or AMP-CP did not alter the systemic hemodynamics (mean blood pressure, 98.8 ± 6.1 versus $101.8 \pm 5.8 \text{ mm Hg}$ before and after 8-SPT and 99.0 ± 3.0 versus $102.0 \pm 3.2 \text{ mm Hg}$ before and after AMP-CP; heart rate, 132.2 ± 6.9 versus $132.4 \pm 6.1 \text{ min}^{-1}$ before and after 8-SPT and 131.8 ± 4.6 versus $132.8 \pm 3.4 \text{ min}^{-1}$ before and after AMP-CP) or the coronary hemodynamic and metabolic parameters (Figures 1 through 3). Before both CBF and CPP were reduced, there were no significant differences in hemodynamic and metabolic parameters among the 3 groups. In untreated dogs, administration of saline did not affect CPP, LER, or FS. However, addition of carvedilol increased VAD(Ado), CBF, LER, and FS, even in the

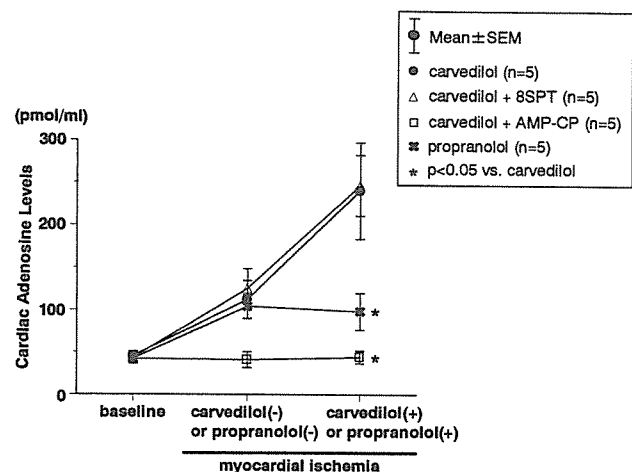


Figure 2. Changes of difference in adenosine levels between coronary venous and arterial blood [VAD(Ado)] in ischemic myocardium. Carvedilol increased VAD(Ado), which was attenuated by an ecto-5'-nucleotidase inhibitor. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

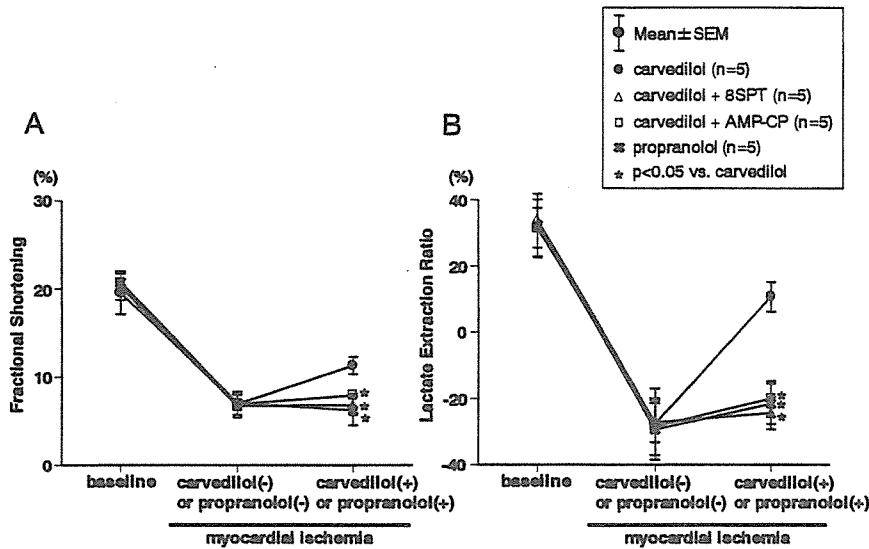


Figure 3. Changes of FS (A) and LER (B) in ischemic myocardium. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

constant low-CPP state, suggesting that myocardial ischemia was improved by carvedilol. These effects of carvedilol were blunted by administration of either 8-SPT or AMP-CP. Unlike carvedilol, an infusion of propranolol did not alter VAD(Ado), CBF, LER, or FS (Figures 1 through 3).

Reduction of Oxidative Stress and Beneficial Effect of Carvedilol in Ischemic Myocardium

In 5 dogs, reduction of CBF caused an increase of VAD(8-Iso-F_{2a}), which was reduced by carvedilol (Figure 4A through 4C). Under these conditions, VAD(Ado) was increased by infusion of carvedilol (Figure 4D). In another 5 dogs, an infusion of SOD did not change either hemodynamic parameters or VAD(8-Iso-F_{2a}) at nonischemic baseline conditions (Figure 4A through 4C). After the reduction of CBF to 50%, VAD(Ado) increased to the level seen in the presence of carvedilol without SOD (Figure 4D), whereas VAD(8-Iso-F_{2a}) did not increase (Figure 4C). Addition of carvedilol did not further attenuate VAD(8-Iso-F_{2a}) or increase VAD(Ado) (Figure 4C and 4D).

Effects of Carvedilol on Infarct Size

Seven of 64 dogs were excluded from analysis because their subendocardial collateral flow was >15 mL · 100 g⁻¹ · min⁻¹, so 57 dogs completed the protocol satisfactorily. Among these 57 dogs, 18 dogs developed ventricular fibrillation at least once, and ventricular fibrillation that matched the exclusion criteria occurred in 15 dogs, so these animals were also excluded from analysis. The numbers of the dogs that met the exclusion criteria of ventricular fibrillation were 2, 2, 0, 2, 3, 3, and 3 in the saline, the DMSO, the carvedilol, the carvedilol+8-SPT, the carvedilol+AMP-CP, the 8-SPT, and the AMP-CP groups, respectively.

Neither aortic blood pressure (≈104 mm Hg) nor heart rate (≈136 min⁻¹) showed any differences among the 7 groups throughout the protocol. The Table shows the area at risk and the endocardial collateral blood flow in the LAD region during myocardial ischemia. There were no significant differences in the area at risk and collateral flow among the 7 groups during myocardial ischemia (Table). Figure 5 shows

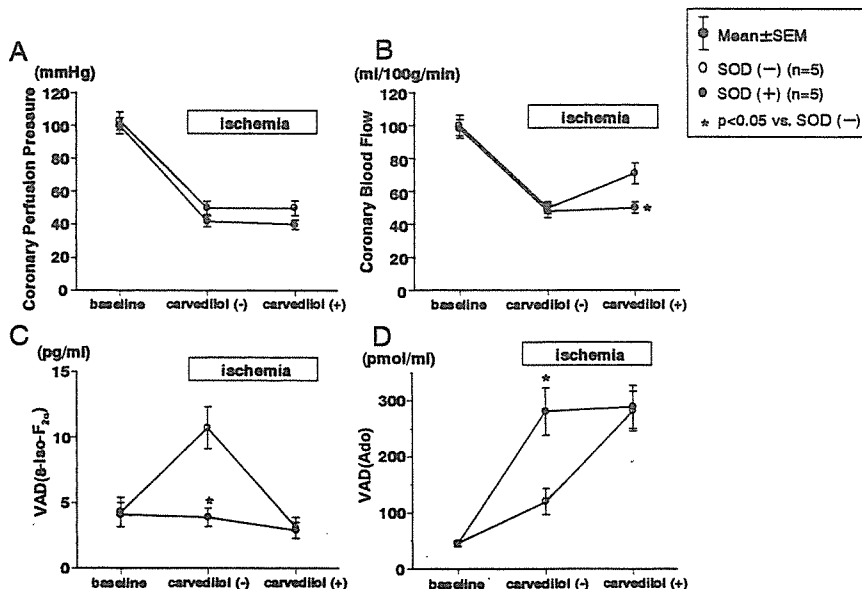


Figure 4. Changes of CPP (A), CBF (B), VAD(8-Iso-F_{2a}) (C), and VAD(Ado) (D) in ischemic myocardium. Statistical significance was tested by ANOVA followed by Bonferroni's test.

Area at Risk and Collateral Blood Flow During Myocardial Ischemia in Each Group

Groups	Risk Area, %	CBF During Myocardial Ischemia, mL·100 g ⁻¹ ·min ⁻¹
1. Control (saline) group	38.9±1.2	7.8±1.3
2. DMSO group	40.2±1.5	8.1±2.3
3. Carvedilol group	41.5±2.2	8.0±2.6
4. Carvedilol group+8-SPT group	39.3±3.3	9.0±2.0
5. Carvedilol group+AMP-CP group	40.9±3.3	8.7±1.9
6. 8-SPT group	43.1±1.9	8.3±1.9
7. AMP-CP group	41.2±2.1	8.2±1.5

Values are expressed as mean±SEM. There were no differences in the area at risk and collateral blood flow in all of the groups. Statistical significance was tested by ANOVA, followed by Bonferroni's test.

that carvedilol decreased infarct size compared with the control groups. This protective effect was completely blocked by either 8-SPT or AMP-CP, suggesting that the reduction of infarct size by carvedilol was attributable to an adenosine-dependent mechanism.

Effect of Carvedilol on Ecto-5'-Nucleotidase Activity in HUVECs

In HUVECs, carvedilol increased ecto-5'-nucleotidase activity by 35.4±8.4% ($P<0.01$) (Figure 6A). Exposure to xanthine and xanthine oxidase decreased ecto-5'-nucleotidase activity, whereas concomitant addition of carvedilol restored ecto-5'-nucleotidase activity to 104.9±8.7% of the baseline levels ($P<0.01$) (Figure 6B). Neither carvedilol nor xanthine and xanthine oxidase had any effect on cytosolic 5'-nucleotidase.

Discussion

In the present study, we demonstrated that carvedilol increases both adenosine release and CBF in ischemic and nonischemic hearts via reduction of oxidative stress and restoration of ecto-5'-nucleotidase activity. We also showed

that carvedilol could limit infarct size and that this effect was attributable to the reduction of oxidative stress and an adenosine- or ecto-5'-nucleotidase-dependent mechanism. These findings suggested that the cardioprotective effect of carvedilol was attributable to an increase of adenosine in ischemic myocardium in addition to its β -blocking action, because propranolol did not mimic this effect.

Influence of Carvedilol on Adenosine Release in Ischemic Hearts

The β -adrenoreceptors in coronary smooth muscle are involved in coronary vasodilation, and their stimulation is thought to increase CBF via the relaxation of vascular smooth muscle and increased myocardial oxygen demand. Therefore, it may seem unusual that a β -blocker like carvedilol would cause coronary vasodilation. There are several possible explanations for the present findings. First, carvedilol itself may cause vasodilation separately from its β -blocking activity. Indeed, although carvedilol does not have a nitroxy moiety, its chemical structure predicts that the drug could also block α_1 -adrenoreceptors,²⁰ which would cause vasodilation. We cannot exclude this possibility, but the role of α_1 -adrenoreceptor blockade in the vasodilatory effect of carvedilol seems likely to be minor, because we have previously reported that blockade of α_1 -adrenoreceptors attenuates adenosine release in ischemic myocardium,²¹ whereas we found that carvedilol caused an increase of adenosine production. Second, carvedilol may increase vasodilatory substances such as NO or adenosine. We demonstrated that carvedilol could increase cardiac adenosine production independently of its β -blocking effect in the present study, because propranolol did not increase CBF under the same circumstances (Figures 1 through 3). Intriguingly, the carvedilol-induced increases in both adenosine release and coronary vasodilation were greater in ischemic heart than in nonischemic heart. There was a significant difference between the influence of carvedilol on coronary vasodilation under nonischemic and ischemic conditions in the present study, because the percent

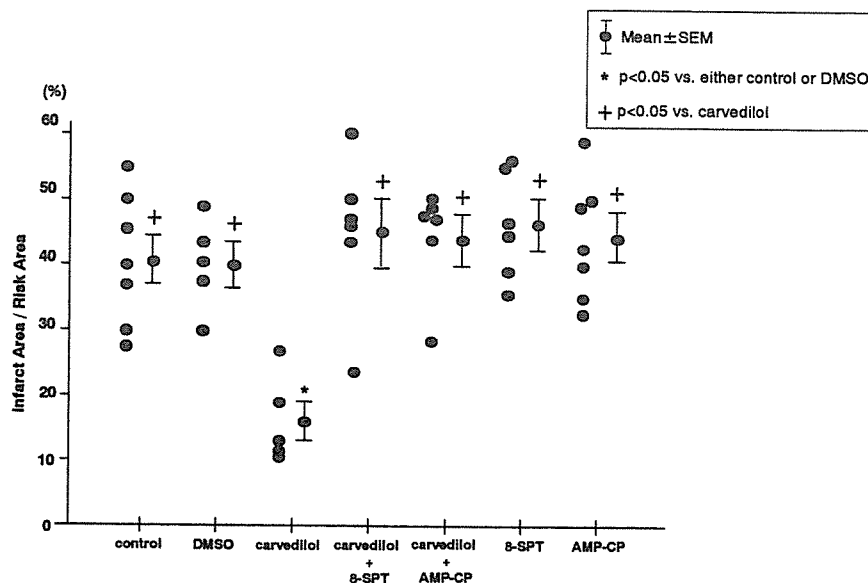


Figure 5. Infarct size as a percentage of area at risk. Infarct size was decreased in carvedilol group compared with control group, and this improvement was blocked by either 8-SPT or AMP-CP. Statistical significance was tested by ANOVA followed by Bonferroni's test.

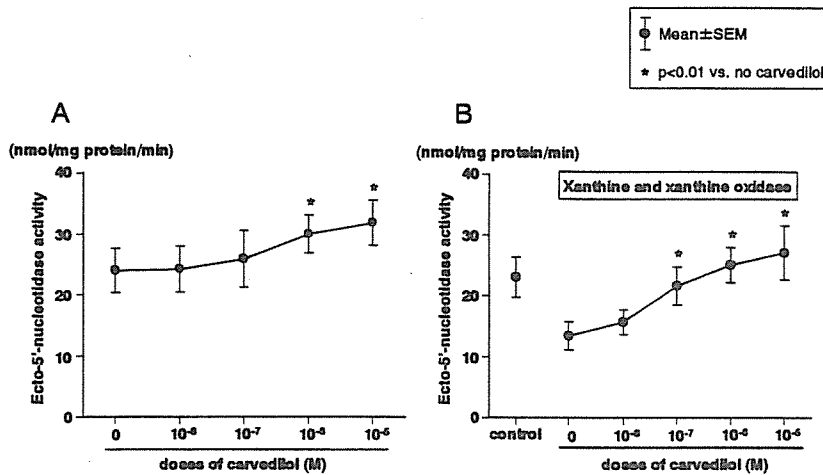


Figure 6. Ecto-5'-nucleotidase activity of HUVECs in presence or absence of carvedilol or xanthine and xanthine oxidase. Statistical significance was tested by ANOVA followed by Bonferroni's test.

increases of CBF in nonischemic and ischemic myocardium were $14.4 \pm 1.1\%$ and $50.6 \pm 10.1\%$ ($P < 0.05$), respectively. One possible explanation is that carvedilol may bind more tightly to β -adrenoreceptors under ischemic conditions than nonischemic conditions, and β -adrenoreceptors are also up-regulated in the ischemic heart,²² which may enhance the adenosine-producing effect of carvedilol. Alternatively, even if carvedilol decreases coronary artery tone in nonischemic heart as well as ischemic heart, the activity of other endogenous vasodilators may decrease to maintain coronary autoregulation. Conversely, the effects of other vasodilators may already be maximal in ischemic hearts, so that carvedilol-induced adenosine release becomes a major determinant of coronary artery tone when adenosine-dependent coronary vasodilation is submaximal. A third possibility is that carvedilol may reduce the levels of substances that attenuate adenosine release and are increased in ischemic myocardium. Because carvedilol is reported to decrease oxidative stress and such stress reduces adenosine production, antioxidant activity of carvedilol may be involved in adenosine-dependent coronary vasodilation and cardioprotection. We showed such evidence in the present study.

In this context, several lines of evidence support the concept that adenosine can markedly attenuate ischemia/reperfusion injury,^{12,23} and we suggest that carvedilol-induced adenosine release is important for cardioprotection.

Mechanism of the Carvedilol-Induced Increase of Cardiac Adenosine

In ischemic hearts, carvedilol caused reduction of oxidative stress and increases in both adenosine release and CBF. Also, in HUVECs under oxidative stress, carvedilol restored ecto-5'-nucleotidase activity to the control level. These findings suggest that carvedilol may eliminate the factors that impaired ecto-5'-nucleotidase activity under ischemic conditions. Oxidative stress is one of these factors. Because oxygen-derived free radicals attenuate the ischemia-induced activation of ecto-5'-nucleotidase, elimination of oxidative stress may increase adenosine release in the ischemic myocardium. We observed that carvedilol could reduce oxidative stress, so this action may explain the present findings. Because ecto-5'-nucleotidase is susceptible to impairment by

oxygen-derived free radicals, it is likely that the beneficial effect of carvedilol on myocardial ischemia in the present study was attributable to its antioxidant activity.

Clinical Relevance and Limitations

Carvedilol has been shown to be effective for treating heart failure.⁵ Its effective clinical dose is about 0.1 to 0.2 $\mu\text{g}/\text{mL}$, and the calculated cardiac concentration of carvedilol in the present study is $\approx 1 \mu\text{g}/\text{mL}$. In dogs, carvedilol at 1 and 4 $\mu\text{g}/\text{mL}$ decreased blood pressure by 9% and 32%, respectively (data not shown), suggesting that the concentration of 1 $\mu\text{g}/\text{mL}$ of carvedilol in canine hearts was comparable to a clinical dose of carvedilol. This difference may be also attributable to species differences, the route of administration of carvedilol, or conscious/anesthetic conditions.

The present study hinted that the mechanism by which carvedilol potentially ameliorates heart failure, especially ischemic heart failure, may be related to adenosine.¹² Carvedilol may have the ability to both antagonize β -adrenoreceptors and increase adenosine release.

Tumor necrosis factor- α is inhibited by both carvedilol and adenosine^{24,25} and has been indicated to have a role in the pathology of congestive heart failure. Because the present study hints that the cardioprotection afforded by carvedilol is adenosine-dependent, it follows that the clinical effects of carvedilol may also be adenosine-dependent. If this hypothesis receives further validation, adenosine and potentiators of adenosine production or adenosine receptor agonists may become candidates for the treatment of heart failure.

Acknowledgments

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SNP Communication

Fourteen Novel Single Nucleotide Polymorphisms in the SLC22A2 Gene Encoding Human Organic Cation Transporter (OCT2)

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Summary: Thirty-three genetic variations including fourteen novel ones were found in the *SLC22A2* gene from 116 Japanese individuals. The novel variations were as follows: 596C>T (MPJ6_OC2003), 602C>T (MPJ6_OC2004), IVS5+20A>G (MPJ6_OC2010), IVS5–84_–83insG (MPJ6_OC2013), IVS6+30T>C (MPJ6_OC2014), IVS6+146G>T (MPJ6_OC2016), IVS6+179G>T (MPJ6_OC2017), IVS6–16delT (MPJ6_OC2018), 1920G>A (MPJ6_OC2022), 2153G>A (MPJ6_OC2026), 2157C>T (MPJ6_OC2028), 2306T>C (MPJ6_OC2031), 2342+5T>C (the last nucleotide number of mRNA + the position in the 3'-flanking region; MPJ6_OC2032) and 2342+127T>C (MPJ6_OC2033). Six variations were located in the exons, four of which were in the 3'-untranslated region (3'-UTR) of exon 11; six were in the introns; and two were in the 3'-flanking region. The frequencies were 0.802 for IVS5–84_–83insG, 0.013 for 602C>T, 0.009 for 596C>T, and 0.004 for the other 11 variations. Among them, 596C>T and 602C>T resulted in amino acid substitutions (Thr199Ile and Thr201Met, respectively).

Key words: *SLC22A2* (OCT2); nonsynonymous alteration; intron; novel SNP

Introduction

Human polyspecific organic cation transporter 2 (OCT2) encoded by *SLC22A2* belongs to a superfamily of transporters, solute carrier family 22 (SLC22), which share a highly homologous 12-transmembrane structure.¹⁾ This family also includes organic cation transporter 1 (OCT1) encoded by *SLC22A1*, which exhibits 70% amino acid similarity to OCT2.²⁾ *SLC22A1* and

SLC22A2 are located adjacently on chromosome 6q26 and both consist of 11 exons,³⁾ suggesting that they evolved from a common ancestor by gene duplication. Both OCT1 and OCT2 translocate a wide variety of endogenous and exogenous organic cations across the plasma membrane in an electrochemical potential-dependent manner.^{4,5)} Although both transporters show an extensive overlap in their substrate specificities, there are distinct differences in tissue distribution. In contrast to OCT1, which is primarily found in the sinusoidal (basolateral) membranes of hepatocytes and, to a lesser extent, in intestinal epithelial cells, OCT2 is mainly found in the basolateral membranes of proximal tubules in the kidney.^{2,4-6)} Based on its properties and tissue distribution, OCT2 is thought to mediate the uptake of organic cations from the blood into renal tubular epithelial cells. Indeed, functional studies using isolated human kidney tubules demonstrate their ability to take

On March 5th, 2004, these variations were not found in the Japanese Single Nucleotide Polymorphisms (JSNP) (<http://snp.ims.u-tokyo.ac.jp/>), dbSNP in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>), or PharmGKB (<http://www.pharmgkb.org/do/>) databases.

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Table 1. Primer sequences used for the analysis of the human *SLC22A2* gene

	Amplified and sequenced region	Forward primer (5' to 3')	Reverse primer (5' to 3')	PCR product (bp)
1st PCR	Exons 1 to 9	CCTCTAAGTACCTGATGTCTGGCTT	CATCAACACAGGTGCTCCATTATGC	20556
	Exons 10 to 11	GCATTTGACTGGCTAAAACCTTGGTG	GTGGGAGGTCTCTCAGAACAAAAGA	10370
2nd PCR	Exon 1	GTGTGTTTTCTCCATAGGGC	CCAGTTATTCTCCAGACAAA	1060
	Exon 2	GCCACTTAACTATACAGTTC	GGAGATTGTGGTCTGTGTGC	350
	Exons 3 to 4	TGCTGATGAAAAGTGCTACC	GGTCTGGAGAGTGAAAGCAA	1796
	Exon 5	CTGAGGAGGGATTTAGCATT	GGCATAGGAGACTGGTGAAG	586
	Exon 6	GTATGGAAAAGTGCTTGCTCT	GTGAAGCCGAGGTTGCCATT	467
	Exon 7	AGCCAGCCACTGAAGTAGAT	GGGCTGTAAAGTCTCTCTTG	618
	Exon 8	CGTGAGAATCTGCTGACATT	ACCCCAGCCTGTAAGTGTGA	449
	Exon 9	GTGATTTCTGAGATTTTGG	GCCACAGACACATCATTACT	413
	Exon 10	GGGTTACAGTCCTCCTTTTC	TTAGAAAGACCTTCTCCACG	652
	Exon 11	CCACCACTCAGAACACATTG	AGAGGTGAAATAGGGCAAGG	1049
	Sequencing	Exon 1 ^a	GTGTGTTTTCTCCATAGGGC ACTCCCCTCTTTGACTTCTG	GGCACCGTGTAGTTCAGTTC CCAGTTATTCTCCAGACAAA
Exon 2		GCCACTTAACTATACAGTTC	GGAGATTGTGGTCTGTGTGC	
Exon 3		TCCCTTTGTGGCTATCAGTC	GAGGAATGCTGAATGAGTTG	
Exon 4		ATTCAGAGAGTTGCGTAGAA	GGTCTGGAGAGTGAAAGCAA	
Exon 5		CTGAGGAGGGATTTAGCATT	GGCATAGGAGACTGGTGAAG	
Exon 6		TTGTTATCCCTATGTGACC	GTGAAGCCGAGGTTGCCATT	
Exon 7		AGCCAGCCACTGAAGTAGAT	GGGCTGTAAAGTCTCTCTTG	
Exon 8		CGTGAGAATCTGCTGACATT	ACCCCAGCCTGTAAGTGTGA	
Exon 9		GTGATTTCTGAGATTTTGG	GCCACAGACACATCATTACT	
Exon 10		GGGTTACAGTCCTCCTTTTC	TTAGAAAGACCTTCTCCACG	
Exon 11 ^a		TTTGGTTTTACAGCCTCCTCA TAACCAGAATACATAGGGGA	TCACCTGTGTTACTGAAAGG AGGTGAAATAGGGCAAGGAA	

^aTwo sets of overlapping primers were used for sequencing of exon 1 and exon 11.

up a number of organic cations with a similar specificity to OCT2.⁷⁾ A wide array of clinically used drugs (*e.g.*, cimetidine, procainamide and metformin), endogenous bioactive amines such as dopamine and norepinephrine, as well as toxic substances [*e.g.*, MPP⁺ (1-methyl-4-phenyl-pyridinium)] were reported to be transported by OCT2.^{2,4,5,8,9)} Thus, OCT2 is thought to have a critical role in the clearance of these compounds.

Recently, several single nucleotide polymorphisms (SNPs) in *SLC22A2* have been identified both in Japanese populations¹⁰⁾ and in ethnically diverse populations,¹¹⁾ and these lists have been published in the JSNP database and the PharmGKB database,¹²⁾ respectively. Furthermore, several SNPs in OCT2 have been reported to affect the transport function of OCT2 *in vitro*.¹¹⁾ These variations may influence renal drug elimination and toxicities *in vivo*. Therefore, studies on genetic variations in *SLC22A2* would be useful for investigating possible correlations between genotypes and phenotypes, such as responsiveness to drug therapy and sensitivity to xenobiotics. In this study, we searched for novel SNPs by sequencing all the exons and the surrounding introns of *SLC22A2* from 116 Japanese individuals. We identified 14 novel variations, including two novel nonsynonymous SNPs located in exon 3.

Materials and Methods

Human genomic DNA samples: All of the 116 arrhythmic patients participating in this study were administered cationic antiarrhythmic drugs at the National Cardiovascular Center. Genomic DNA was extracted from blood leukocytes and was used as a template in the polymerase chain reaction (PCR). The ethical review boards of the National Cardiovascular Center and National Institute of Health Sciences approved this study. Written informed consent was obtained from all participating subjects.

PCR conditions for DNA sequencing: First, the entire *SLC22A2* gene was divided into two regions (from exon 1 to exon 9 and from exon 10 to 11), and each region was amplified from 100 ng of genomic DNA using 1.25 units of Z-Taq (Takara Shuzo, Tokyo, Japan) with 0.2 μ M primers listed in **Table 1** (1st PCR). The first PCR conditions were 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec. Next, each exon was amplified by Ex-Taq (0.625 units) (Takara Shuzo) with an appropriate set of *SLC22A2*-specific primers (2nd PCR; 0.2 μ M) designed in the introns, as listed in **Table 1**. The second round PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min,

Table 2. Summary of variations in the *SLC22A2* gene detected in Japanese arrhythmic patients

SNP ID	NCBI ^b	JSNPs ^c	Location	Accession number NT_007422.12	position		Nucleotide change and flanking sequence (5' to 3')	Amino acid change	Frequency
					From the translational initiation site or from the nearest exon				
MPJ6_OC2001	rs624249	ssj0008486	Exon 1	2966741	390 ^d		GGTGTACGAGACG/TCCTGGCTCGTCC	Thr130Thr	0.138
MPJ6_OC2002	rs2774230	ssj0008488	Intron 2	2964955	IVS2 + 32		GTGCAATTTAAAG/CAATCCCAAGGT		0.177
MPJ6_OC2003 ^a			Exon 3	2958998	596 ^d		CCATTTCCCAAC/TCTATACGTGGAT	Thr199Ile	0.009
MPJ6_OC2004 ^a			Exon 3	2958992	602 ^d		CCCCAACCTATAC/TGTGGATGTTAAT	Thr201Met	0.013
MPJ6_OC2005	rs316016	IMS-JST027183, ssj0008491	Intron 3	2958841	IVS3 + 80		GGCGGCATGCAG/AGAAAAGGACCC		0.246
MPJ6_OC2006	rs316017	IMS-JST027184, ssj0008492	Intron 3	2957874	IVS3 - 117		TCAGAGAGTTGCA/GTAGAATATCTA		0.246
MPJ6_OC2007	rs316018	IMS-JST027185, ssj0008493	Intron 3	2957835	IVS3 - 78		TTTTGAGGTTGGC/TGTTCTAGTTCC		0.246
MPJ6_OC2008	rs316019	IMS-JST027186	Exon 4	2957623	808 ^d		CAGTTCACAGTTG/TCTCTGCCAACT	Ala270Ser	0.168
MPJ6_OC2009	rs2279463	IMS-JST027187, ssj0008494	Intron 4	2955730	IVS4 - 59		ATTATAAAAAAAAT/CGGGGATGGGGT		0.078
MPJ6_OC2010 ^a			Intron 5	2955537	IVS5 + 20		AGGCCTTAAGTA/GTCAAATCAGGGG		0.004
MPJ6_OC2011	rs3219195		Intron 5	2955529, 2955528	IVS5 + 28, + 29		AAAGTATCAAATCAG/-GGGATGGAGAA		0.078
MPJ6_OC2012	rs316021	IMS-JST027188, ssj0008495	Intron 5	2955382	IVS5 + 175		ATTTGCGTTCCCA/GTGGGAGAAATAT		0.259
MPJ6_OC2013 ^a			Intron 5	2954002, 2954001	IVS5 - 84, - 83		CGCTCAGGACGG/-GTAAGAAATATAT		0.802
MPJ6_OC2014 ^a			Intron 6	2953782	IVS6 + 30		CTTTGAAATGCCI/CCCAAATTTGTTT		0.004
MPJ6_OC2015	rs617217	ssj0008496	Intron 6	2953697	IVS6 + 115		TGACGGTGGCAC/GCAAATGTTGGTTG		0.198
MPJ6_OC2016 ^a			Intron 6	2953666	IVS6 + 146		GTAGGTTTTCCCTG/TATCGTGTGTTTC		0.004
MPJ6_OC2017 ^a			Intron 6	2953633	IVS6 + 179		ATATTAAAGATCGG/TTGTAGCCCTAA		0.004
MPJ6_OC2018 ^a			Intron 6	2952175	IVS6 - 16		CTTGACCTGAACT/-CTCCTCTTTGCT		0.004
MPJ6_OC2019	rs8177518		Intron 7	2950810	IVS7 - 35		TGAGGAATCATCT/CGTGTACGGATAA		0.065
MPJ6_OC2020	rs3839344	IMS-JST156304, ssj0005336	Intron 9	2933263, 2933262	IVS9 - 86, - 85		TGCTAAAAAAA-/AGTTTTAAACAAA		0.802
MPJ6_OC2021	rs316003	ssj0008502	Exon 10	2933173	1506 ^d		GCTTTCAGGCGTA/GCTTTGGCTTGGTT	Val502Val	0.198
MPJ6_OC2022 ^a			Exon 11 (3'-UTR)	2925553	1920 ^d		GGTTTTCTGGAGG/AGTTTTTTTTTCCA		0.004
MPJ6_OC2023	rs3127594	ssj0005340	Exon 11 (3'-UTR)	2925445	2028 ^d		AAAAATAGAAAAAA/TGTTGTGAAAAACA		0.017
MPJ6_OC2024	rs3103353	ssj0005341	Exon 11 (3'-UTR)	2925417	2056 ^d		AAGTTGGGAGAGG/AAGCATCTATTTT		0.017
MPJ6_OC2025	rs3127593	ssj0005342	Exon 11 (3'-UTR)	2925344	2129 ^d		TGTCAGAAATTTT/AAAGATAGGCCTTT		0.017
MPJ6_OC2026 ^a			Exon 11 (3'-UTR)	2925320	2153 ^d		TCAGTAACACACAGG/ATGAAGAAAATTT		0.004
MPJ6_OC2027	rs2450975	IMS-JST156307, ssj0008505	Exon 11 (3'-UTR)	2925316	2157 ^d		TAAACACAGGTGAC/AGAAAATTTTTAAA		0.168
MPJ6_OC2028 ^a			Exon 11 (3'-UTR)	2925316	2157 ^d		TAAACACAGGTGAC/AGAAAATTTTTAAA		0.004
MPJ6_OC2029	rs694812	IMS-JST183810	Exon 11 (3'-UTR)	2925231	2242 ^d		TACAACTTAAGTA/GATCATGAGGTCC		0.151
MPJ6_OC2030	rs3127592		Exon 11 (3'-UTR)	2925185	2288 ^d		GCATGAATGTGA/GTTTTCTACAAAA		0.017
MPJ6_OC2031 ^a			Exon 11 (3'-UTR)	2925167	2306 ^d		TACAAAAGACCTT/CGAAGATGATTTCA		0.004
MPJ6_OC2032 ^a			3'-flanking	2925126	2342 + 5 ^c		TTATAAAAACCAAT/CGATGATCAATTT		0.004
MPJ6_OC2033 ^a			3'-flanking	2925004	2342 + 127 ^c		TCATAAGACTTAT/CAATGAAAAACAAA		0.004

^aFourteen novel variations detected in our study.^bThe rs number in NCBI dbSNP.^cThe ID number in the JSNP database.^dA of the translation initiation codon ATG is numbered 1.^eThe last nucleotide number of mRNA + the position in the 3'-flanking region.

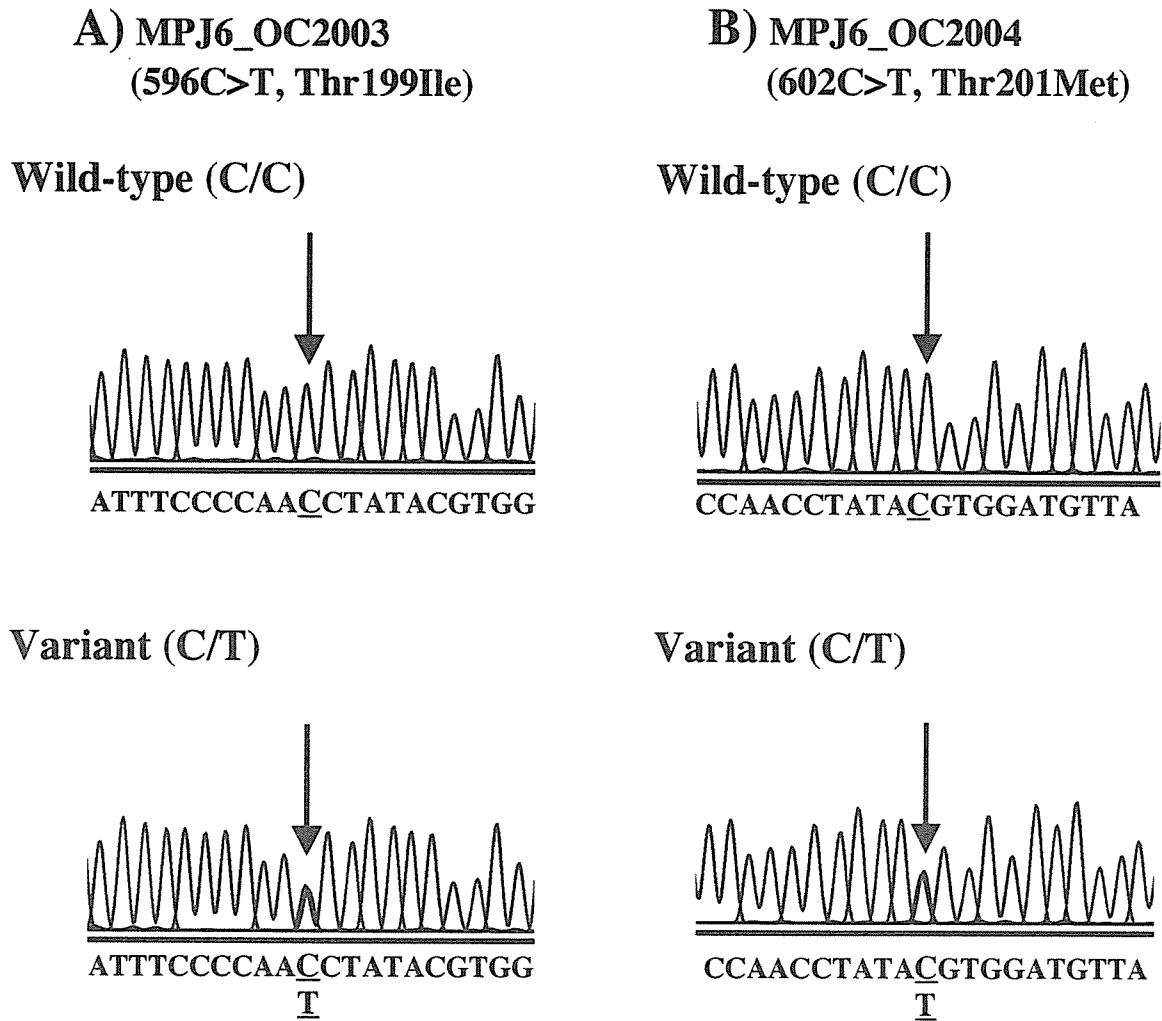


Fig. 1. Electropherograms (sense strands) for the novel nonsynonymous SNPs in *SLC22A2*. (A) MPJ6_OC2003 (wild-type 596C/C; variant 596C/T). (B) MPJ6_OC2004 (wild-type 602C/C; variant 602C/T). The first A of the translation initiation codon ATG is defined as position 1. Arrows indicate the position of the nucleotide changes.

and then a final extension for 7 min at 72°C. The PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and were directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in **Table 1** (Sequencing). The excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). All novel SNPs were confirmed by repeated sequence analyses on PCR products generated by a new genomic DNA amplification.

Results and Discussion

We sequenced all the exons (exons 1~11) and their flanking regions of *SLC22A2* for 116 Japanese arrhythmic patients, and found 33 variations, including 14

novel ones (**Table 2**). In this paper, genomic and cDNA sequences of *SLC22A2* obtained from GenBank (NT_007422.12 and NM_003058.2, respectively) were used as the reference sequences. The novel variations were 596C>T in exon 3 (the A of the translation start codon is numbered +1 in the cDNA sequence; MPJ6_OC2003), 602C>T in exon 3 (MPJ6_OC2004), IVS5+20A>G in intron 5 (20 nucleotides downstream from exon 5; MPJ6_CS2010), IVS5-84-83insG in intron 5 (from 83 to 84 nucleotides upstream from exon 6; MPJ6_OC2013), IVS6+30T>C in intron 6 (MPJ6_OC2014), IVS6+146G>T in intron 6 (MPJ6_OC2016), IVS6+179G>T in intron 6 (MPJ6_OC2017), IVS6-16delT in intron 6 (MPJ6_OC2018), 1920G>A in exon 11 (MPJ6_OC2022), 2153G>A in exon 11 (MPJ6_OC2026), 2157C>T in exon 11 (MPJ6_OC2028), 2306T>C in exon 11

(MPJ6_OC2031), 2342+5T>C in the 3'-flanking region (5 nucleotides downstream from the last nucleotide of mRNA; MPJ6_OC2032) and 2342+127T>C in the 3'-flanking region (MPJ6_OC2033). The frequencies were 0.802 for IVS5-84-83insG, 0.013 for 602C>T, 0.009 for 596C>T and 0.004 for the other 11 variations. Nineteen variations we detected were already reported and publicized in the dbSNP database and/or the JSNP database. Their ID numbers in the databases are also given in **Table 2**.

Recently, twenty-eight polymorphisms in *SLC22A2* were identified in several ethnically diverse populations, including 30 Asian-Americans, by Leabman *et al.*¹¹⁾ and published in the PharmGKB database.¹²⁾ Among them, six variations, 390G>T (Thr130Thr) (MPJ6_OC2001), IVS2+32G>C (MPJ6_OC2002), 808G>T (Ala270Ser) (MPJ6_OC2008), IVS5+28_+29delAG (MPJ6_OC2011), IVS7-35T>C (MPJ6_OC2019) and 1506A>G (Val502Val) (MPJ6_OC2021) were also detected in our study. The other SNPs reported in their study were not detected in our study. The 808G>T (Ala270Ser) SNP was the only nonsynonymous SNP found commonly in both their study¹¹⁾ and ours (**Table 2**) at an allele frequency greater than 10%. The frequency for this SNP was 0.168 in our study, which was comparable to those in Caucasians (0.157), African-Americans (0.110)¹¹⁾ and the JSNPs database (0.120). However, it was higher than that in 30 Asian-Americans (0.086).¹¹⁾ This difference in frequency between Asian-Americans and Japanese may be due to the numbers of subjects analyzed. Leabman *et al.*¹¹⁾ reported that the 808G>T change exhibits a slight increase in the Km value of the prototypical organic cation, MPP⁺, and a significant increase in the Ki value of tetrabutylammonium (TBA) when expressed in *Xenopus laevis* oocytes. They suggested that the 808G>T (Ala270Ser) change might influence drug response by altering the renal elimination of xenobiotics *in vivo*.

We identified two novel nonsynonymous SNPs. These SNPs were as follows:

- 1) SNP, MPJ6_OC2003; GENE NAME, *SLC22A2*; ACCESSION NUMBER, NT_007422.12; LENGTH, 25 bases; 5'-CCATTCCCCAAC/TCTATACGTGG-AT-3'
- 2) SNP, MPJ6_OC2004; GENE NAME, *SLC22A2*; ACCESSION NUMBER, NT_007422.12; LENGTH, 25 bases; 5'-CCCCAACCTATAC/TGTGGATGTT-AAT-3'.

The 596C>T (Thr199Ile) (MPJ6_OC2003) change was found in two heterozygous subjects. Another SNP, 602C>T (Thr201Met) (MPJ6_OC2004), was found in 3 different heterozygous subjects. The electropherograms of these two novel nonsynonymous SNPs are shown in **Fig. 1**. Both SNPs were located in exon 3, in a short

extracellular loop between the third transmembrane domain (TMD3) and the fourth transmembrane domain (TMD4). These two threonine residues are not conserved among the human OCT family (OCT1, OCT2 and OCT3). Functional significances of these amino acid substitutions on transport activity are currently unknown. Further analyses of these two SNPs are needed. The other 12 novel SNPs were located in introns, the 3'-UTR region or 3' flanking region. At position 2157, bi-directional changes, C>T (MPJ6_OC2028) and C>A (MPJ6_OC2027), were found. The biological significance of these 12 SNPs also remains to be evaluated.

In conclusion, we identified 33 variations including 14 novel ones in *SLC22A2* of Japanese subjects. Two novel SNPs resulted in amino acid substitutions. The current data may be useful for haplotype analysis and pharmacogenetic studies on OCT2.

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SNP Communication

Novel Nonsynonymous Single Nucleotide Polymorphisms in the CYP2D6 Gene

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Full text of this paper is available at <http://www.jssx.org>

Summary: Cytochrome P450 (CYP) 2D6 is an important drug-metabolizing enzyme, and its gene is known to be highly polymorphic. Here, we report five novel nonsynonymous single nucleotide polymorphisms (SNPs), and 65 other sequence variations detected from the gene coding for cytochrome P450 (CYP) 2D6 in 254 Japanese subjects. Two of the novel nonsynonymous SNPs were associated with the *10 key SNP, C100T. Among the 65 variations, 23 were novel, including 12 SNPs in 5'-flanking, 1 in 5'-untranslated, and 10 in intronic regions.

The nonsynonymous SNPs in the *CYP2D6* gene were as follows: 73 C>T (Arg25Trp, exon 1), 972 C>T (Ala90Val, exon 2), 1611 T>A (Phe120Ile, exon 3), 1720 A>C (Glu156Ala, exon 3), 3172 A>C (Glu334Ala, exon 7). The SNPs, 73C>T, 972 C>T, 1611 T>A, 1720 A>C and 3172 A>C were linked with *10, *1, *10, *1 and *2, respectively.

Key words: CYP2D6; SNP; nonsynonymous SNP

Introduction

Cytochrome P450 (CYP) 2D6 encoded by the *CYP2D6* gene is clinically important since it metabolizes a wide variety of clinical drugs including antiarrhythmic and psychiatric drugs, as well as endogenous compounds.¹⁾ The *CYP2D6* gene is highly polymorphic, and nearly 50 different alleles or haplotypes have been described (References 2 and 3 for review and <http://www.imm.ki.se/CYPalleles/cyp2d6.htm>). These include a deletion of the entire gene, amino acid substitutions, splicing defects, and insertions and deletions of nucleotide(s) resulting in frameshifts in the open reading

frame.

During the course of our discovering new *CYP2D6* variants by direct sequencing of leukocyte DNA of Japanese subjects who were administered antiarrhythmic drugs, we detected 70 sequence variations including 5 novel nonsynonymous single nucleotide polymorphisms (SNPs).

Materials and Methods

Human genomic DNA samples: Total genomic DNA was extracted from blood leukocytes from 254 Japanese individuals, who were administered antiarrhythmic drugs and/or β -blockers, and was used for DNA sequence analysis. The ethics committees of both the National Cardiovascular Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all subjects participating in this study.

Polymerase chain reaction (PCR) conditions for sequencing: 5'-Flanking and exonic regions of the *CYP2D6* gene were separately analyzed by a PCR-based

On April 30, 2004, these SNPs were not found in "dbSNP in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>)", or "A database for CYP Allele Nomenclature (<http://www.imm.ki.se/CYPalleles/>)".

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Table 1. Primers used for the amplification and sequencing of 5'-flanking region and all exons of *CYP2D6* gene

5'-Flanking region: 1st PCR			
2D6-CYP505F	CACTGGCTCCAAGCATGGCAG		
2D6-5'2722R	GCTCGGACTACGGTCATCAC		
5'-Flanking region: 2nd PCR and sequencing primers			
2nd PCR primer		Sequencing primer	
2D65'F1s	AGGTCACTCTGGAGTGGGC	2D65'F1s	AGGTCACTCTGGAGTGGGC
2D65'R1s	AGTCCACATGCAGCAGGTTGC	2D65'R1s	AGTCCACATGCAGCAGGTTGC
2D6-CYP505F	CACTGGCTCCAAGCATGGCAG	2D6-CYP505F	CACTGGCTCCAAGCATGGCAG
2D65'R2s	TGCAATGATGCAATCTCGGCTC	2D65'R2s	TGCAATGATGCAATCTCGGCTC
2D65'F3	ATGTTGTACAGGCTGGGGC	2D65'F3	ATGTTGTACAGGCTGGGGC
2D65'R3	GGCTCATACACCCTAATGTAGTC	2D65'R3	GGCTCATACACCCTAATGTAGTC
2D65'F7	CTGAAGTCAGGAGTTCAAGAC	2D65'F4	AGCTACTTAGGAGGCTGAGGC
2D65'R4	CGGGCTATGGCTTCTTGTGTTGC	2D65'R4s2	GGAGACGGAGATTTCCTCTTGTG
		2C65'F6s	AAGAATTAGGCTGGGTGGTGC
		2C65'R5s	CTTTAGACAGGGTCTCACTC
		2D65'F4s3	AAAGCAACATATCCTGAACAAAGG
		2D65'R4	CGGGCTATGGCTTCTTGTGTTGC
CYP2D6 exons: 1st PCR			
2D6-Dlow	CAGGCATGAGCTAAGGCACCCAGAC		
2D6-Dup	CACACCGGGCACCTGTA CTCTCA		
2D6-DPKup	GTTATCCCAGAAGGCTTTGCAGGCTTCA		
2D6-DPKlow	GCCGACTGAGCCCTGGGAGGTAGGTA		
CYP2D6 exons: 2nd PCR and sequencing primers			
2nd PCR primer		Sequencing primer	
2D6Ex1-2F1	CAGAGGAGCCCATTGTTGGTAGTGAGGCAGGT	2D6Ex1F3s	GAGGCAGGTATGGGGCTA
2D6Ex1-2R1	GGTCCCACGGAAATCTGTCTCTGT	2D6Ex1R4s	TCAGCAGAAGGGACTTTGTAC
		2D6Ex2F2s	TCATACCTGGGTGACGCATCC
		2D6Ex2R2s	GAAATCTGTCTCTGTCCCCAC
2D6Ex3-4F1	CACGCGCACGTGCCCGTCCCA	2D6Ex3F4s	CACGTGCCCGTCCAC
2D6Ex3-4R1	CTCTCGCTCCGCACCTCGCGCAGA	2D6Ex3R1s	TTGCTCACGGCTTTGTCC
		2D6Ex4F1s	ACAAAGCGGGAAGTGGGA
		2D6Ex4R3s	GCAGAAAGCCCCGACTCC
2D6Ex5-6F1	GGACTCTGTACCTCCTATCCACGTCA	2D6Ex5F2s	TTGGTGAGGTGAGTGGTAAGGA
2D6Ex5-6R1	CCCTCGGCCCTGCACTGTTCCCA	2D6Ex5R1s	CCCCAAATGACCTCCAAT
		2D6Ex6F1s	AGGAGGAATGAGGGGAGG
		2D6Ex6R3s	GCACTGTTTCCAGATGG
2D6Ex7F2	TCTGGGAAACAGTGCAGGGCCGAGGG	2D6Ex7F2s	ATCCTGTAAGCCTGACCTCC
2D6Ex7R2	TCTGGCAGGTCTGGTTGTCTCCC	2D6Ex7R2s	CAGTGTGGTGGCATTGAGGA
2D6Ex8-9F1	GGGAGACAAACCAGGACCTGCCAGA	2D6Ex8F2s	GTCCCCGTGTGTTTGGT
2D6Ex8-9R1	CATCTGCTCAGCCTCAACGTACCCCTGTCT	2D6Ex8R1s	GGGAGGTGAAGAAGAGGAAG
		2D6Ex9F2s	CCTTCTGCCTTCTCAGC
		2D8Ex9R2s	GGTAAGCAGGAATGAGGCAG

method. Primer sets used for the amplification and sequencing of the 5'-flanking region and all exons are described in **Table 1**. Two-step PCR reactions were used for the amplification of the 5'-flanking region of the *CYP2D6*.

The first-step PCR amplification of the 5'-flanking region was conducted in a reaction mixture (100 μ L) containing 1 \times Ex Taq buffer, 3.0 mM MgCl₂, 5.0 mM

dNTPs, 1 unit of Ex Taq polymerase (TaKaRa Shuzo, Kyoto, Japan), and 0.5 μ M of each primer. The PCR amplification conditions were as follows: 94°C for 5 min, followed by 30 cycles consisting 94°C for 30 s, 64°C for 1 min, 72°C for 3 min, then 72°C for 7 min, followed by 4°C. For the analysis of each *CYP2D6* exon and surrounding introns, a DNA fragment containing all *CYP2D6* exons was amplified using the Dup, Dlow,

Table 2. SNPs detected in the *CYP2D6* gene in Japanese arrhythmic patients

SNP ID	SNP name	CYP Allele Nomenclature or NCBI dbSNP or Reference	Location	Position		SNP and the surrounding sequence (5' to 3')	Amino acid change	Frequency
				Position in accession number, M33388	Relative to the transition initiation site ^a or from the nearest exon			
MPJ6_2D6041		Ref. 8	5'-Flanking	NA ^b	-1770	GTGGATGATCCCG/ATAGAAAGTCCAGA		0.1784
MPJ6_2D6042		Novel	5'-Flanking	13	-1607	GAATTCAAAGACCA/CGCCTGGACAACT		0.0023
MPJ6_2D6043		*2, Ref. 8	5'-Flanking	36	-1584	CTTGGAAAGAACCC/GGGTCTCTACAAA		0.1596
MPJ6_2D6044		*10	5'-Flanking	194	-1426	CTACTGAAAATAC/TAAAAAGTAGAC		0.3192
MPJ6_2D6046		*2, *10	5'-Flanking	385	-1235	AAAAAAAAGAG/GATTAGGCTGGGT		— ^c
MPJ6_2D6047		*10, Ref. 8	5'-Flanking	620	-1000	GTGGAGGAGGACG/AACCCCTCAGGCAG		0.3099
MPJ6_2D6048		Novel	5'-Flanking	667	-953	CTGGGCAAGGGC/TCTTCCGGCTACC		0.0070
MPJ6_2D6049		Novel	5'-Flanking	672	-948	GCAAGGCCCTCC/AGGCTACCAACTG		0.0023
MPJ6_2D6050		Novel	5'-Flanking	802	-818	CAGAAAACCTCCG/CGCATGGCTGGGA		0.0047
MPJ6_2D6051		rs10080991, *2	5'-Flanking	880	-740	GAGAAATGTGCC/TCTAAAGTGTCAAT		0.1831
MPJ6_2D6052		Novel	5'-Flanking	902	-718	AGTGTGAGTCTGT/CGTATGTGAAAT		0.0023
MPJ6_2D6053		*2, rs769256	5'-Flanking	942	-678	GTGATTTCTGCG/ATGTGTAATCGTG		0.1831
MPJ6_2D6054		Novel	5'-Flanking	1051	-569	CATGTCAAAGAGTG/ACAAGGTGAAAGTG		0.0023
MPJ6_2D6055		Novel	5'-Flanking	1122	-498	CCCAGGTAAGTGC/ACAGTACAGATA		0.1315
MPJ6_2D6058		Novel	5'-Flanking	1252	-368	GGGAGGTGATGG/CCCCGGTCCACTG		0.0023
MPJ6_2D6059		rs5758604	5'-Flanking	1266	-354	CGGGTCCACTGAA/GACCTGGTTATC		0.0023
MPJ6_2D6060		Novel	5'-Flanking	1388	-232	AGCAGGAAGCAGG/CGGCAAGAACCTC		0.0047
MPJ6_2D6062		Novel	5'-Flanking	1395	-225	AGCAGGGGCAAGA/GACCTCTGGAGCA		0.0047
MPJ6_2D6063		Novel	5'-Flanking	1482	-138	ACAGCAGAGGGCA/GAAGGCCATCATC		0.0047
MPJ6_2D6064		Novel	5'-Flanking	1523	-97	GAAGGCTCACGGC/ACTCGGTGTGCTG		0.0023
		Novel	Exon 1	1534	-86	CGTCCGGTGTGCT/CGAGAGTGTCCCTG		0.0023
		Novel (*47)	(5'-UTR)					
MPJ6_2D6065		Novel (*47)	Exon 1	1692	73	GACCTGATGCACC/TGGGGCCAAACGGCT	R25W	0.0023
MPJ6_2D6066		*22, *44, Ref. 9	Exon 1	1701	82	CACCGCGCCAAAC/TGCTGGGTGCAC	R28C	0.0023
MPJ6_2D6001		*10	Exon 1	1719	100	GCTGCACGCTACC/TCACCAGGCCCCC	P34S	0.3122
MPJ6_2D6067		Novel	Intron 1	1832	213 (IVS1 + 33)	GGGGCCAGAGGT/AGCTGAGGCTCCC		0.0047
MPJ6_2D6002		*2, rs1080995, Ref. 6	Intron 1	1833	214 (IVS1 + 34)	GGGGCCAGAGGTG/CCTGAGGCTCCC		0.1831
MPJ6_2D6003		*2, rs1080996, Ref. 6	Intron 1	1840	221 (IVS1 + 41)	GAGGTGCTGAGGC/ATCCCCTACCAGA		0.1831
MPJ6_2D6004		*2, rs1080997, Ref. 6	Intron 1	1842	223 (IVS1 + 43)	GGTGTGAGGTC/GCCCTACCAGAAG		0.1831
MPJ6_2D6005		*2, rs1080998, Ref. 6	Intron 1	1846	227 (IVS1 + 47)	CTGAGGCTCCCCT/GACCAGAAACAAA		0.1831
MPJ6_2D6006		*2, rs1080999, Ref. 6	Intron 1	1851	232 (IVS1 + 52)	CTCCCTACCAG/CAAGCAAAACATGG		0.1831
MPJ6_2D6007		*2, rs1080999, Ref. 6	Intron 1	1852	233 (IVS1 + 53)	GTCCTACCAGA/CAGCAAAACATGGA		0.1831
MPJ6_2D6008		*2, rs1081000, Ref. 6	Intron 1	1864	245 (IVS1 + 65)	AAGCAAAACATGGA/GTGGTGGGTGAAA		0.1831
MPJ6_2D6068		rs1966157	Intron 1	1889	270 (IVS1 + 90)	CCACAGGCTGGAC/TCAGAAAGCCAGGC		0.0047
MPJ6_2D6009		rs1081002, rs1966156	Intron 1	1929	310 (IVS1 + 130)	GGTTTGGGGGACG/TCCTGGAGAAGGG		0.4977
MPJ6_2D6069		rs1966155	Intron 1	1936	317 (IVS1 + 137)	GGGACGTCCTGGA/GGAAAGGGCATTTA		0.0047
MPJ6_2D6070		rs1966154	Intron 1	1942	323 (IVS1 + 143)	TCCTGGAGAAGGG/ACATTTATACATG		0.0047
MPJ6_2D6071		Novel	Intron 1	2270	651 (IVS1 - 233)	ACTGGGGCCTCTG/TGGCAATTTGGGT		0.0023
MPJ6_2D6010		Ref. 7	Intron 1	2365	746 (IVS1 - 138)	ACCCCGGCCCCAC/GATCAGGAGGCT		0.1878

SNP name		Position		SNP and the surrounding sequence (5' to 3')	Amino acid change	Frequency
SNP ID	CYP Allele Nomenclature or NCBI dbSNP or Reference	Location	Position in accession number, M33388			
MP16_2D6012	rs769261, Ref. 7	Intron 1	2462	843 (IVS1 - 41)		0.5070
MP16_2D6073	Novel (*48)	Exon 2	2591	972	A90V	0.0023
MP16_2D6013	*10, rs030864	Exon 2	2658	1039		0.3028
MP16_2D6074	Novel (*49)	Exon 3	3230	1611	F120I	0.0047
MP16_2D6014	*2, *10 rs1058164	Exon 3	3280	1661		0.4930
MP16_2D6075	Novel (*50)	Exon 3	3339	1720	E156A	0.0023
MP16_2D6015	*14	Exon 3	3377	1758	G169R	0.0070
MP16_2D6076	Novel	Intron 3	3383	1746 (IVS3 + 6)		0.0023
MP16_2D6016	Ref. 7	Intron 3	3409	1790 (IVS3 + 32)		0.0117
MP16_2D6078	Novel	Intron 4	3922	2303 (IVS4 - 138)		0.0141
MP16_2D6079	Novel	Intron 4	3981-3992	2361-2373		0.0023
			12bp deletion	GGCCTGAGACTT		
MP16_2D6017	rs2267444, Ref. 7	Intron 4	4043	2424 (IVS4 - 17)		0.0188
MP16_2D6018	Ref. 7	Intron 4	4043	2424 (IVS4 - 17)		0.0117
MP16_2D6088	rs1058169	Exon 5	4089	2470		0.0023
MP16_2D6019	*21	Exon 5	4192	2573	Frameshift	0.0070
MP16_2D6020	Ref. 7	Intron 5	4282	2663 (IVS5 + 46)		0.0469
MP16_2D6080	Novel	Intron 5	4339	2720 (IVS5 - 88)		0.0023
MP16_2D6021	Ref. 7	Intron 5	4378	2759 (IVS5 - 49)		0.0141
MP16_2D6022	*2, rs4994476	Exon 6	4557	2850	R296C	0.1831
MP16_2D6081	*44, Ref. 9	Intron 6	4569	2950 (IVS6 + 1)	Splicing defect	0.0023
MP16_2D6082	Novel (*51)	Exon 7	4791	3172	E334A	0.0023
MP16_2D6023	rs9611741, Ref. 7	Intron 7	4857	3384 (IVS7 + 40)		0.5047
MP16_2D6024	Ref. 7	Intron 7	5027	3408 (IVS7 + 64)		0.0023
MP16_2D6083	Novel	Intron 7	5094	3475 (IVS7 + 131)		0.0023
MP16_2D6084	Novel	Intron 7	5201	3582 (IVS7 + 238)		0.3146
MP16_2D6085	*2	Intron 7	5203	3584 (IVS7 + 240)		0.1876
MP16_2D6086	Novel	Intron 7	5222	3603 (IVS7 - 196)		0.0023
MP16_2D6025	rs4987144	Intron 7	5409	3790 (IVS7 - 9)		0.1831
MP16_2D6087	Novel	Intron 8	5698	4019 (IVS8 - 20)		0.0023
MP16_2D6026	*18	Exon 9	5744-5752	4125-4133	ins VPT	0.0023
				TGTGCCACTGGACAGCCCCGGC		
MP16_2D6027	*2, *10, rs2103556	Exon 9	5799	4180	S486T	0.4977
MP16_2D6038	*5	Whole Deletion				0.0352
				CTTTCCTGGTGAG/CCCCATGCCCCCTA		

^a A of the translation initiation codon ATG is numbered +1.

^b This SNP position is at nucleotide 2431 in a reference sequence, AY545216.

^c Accurate frequency could not be evaluated due to the surrounding repeat sequences.

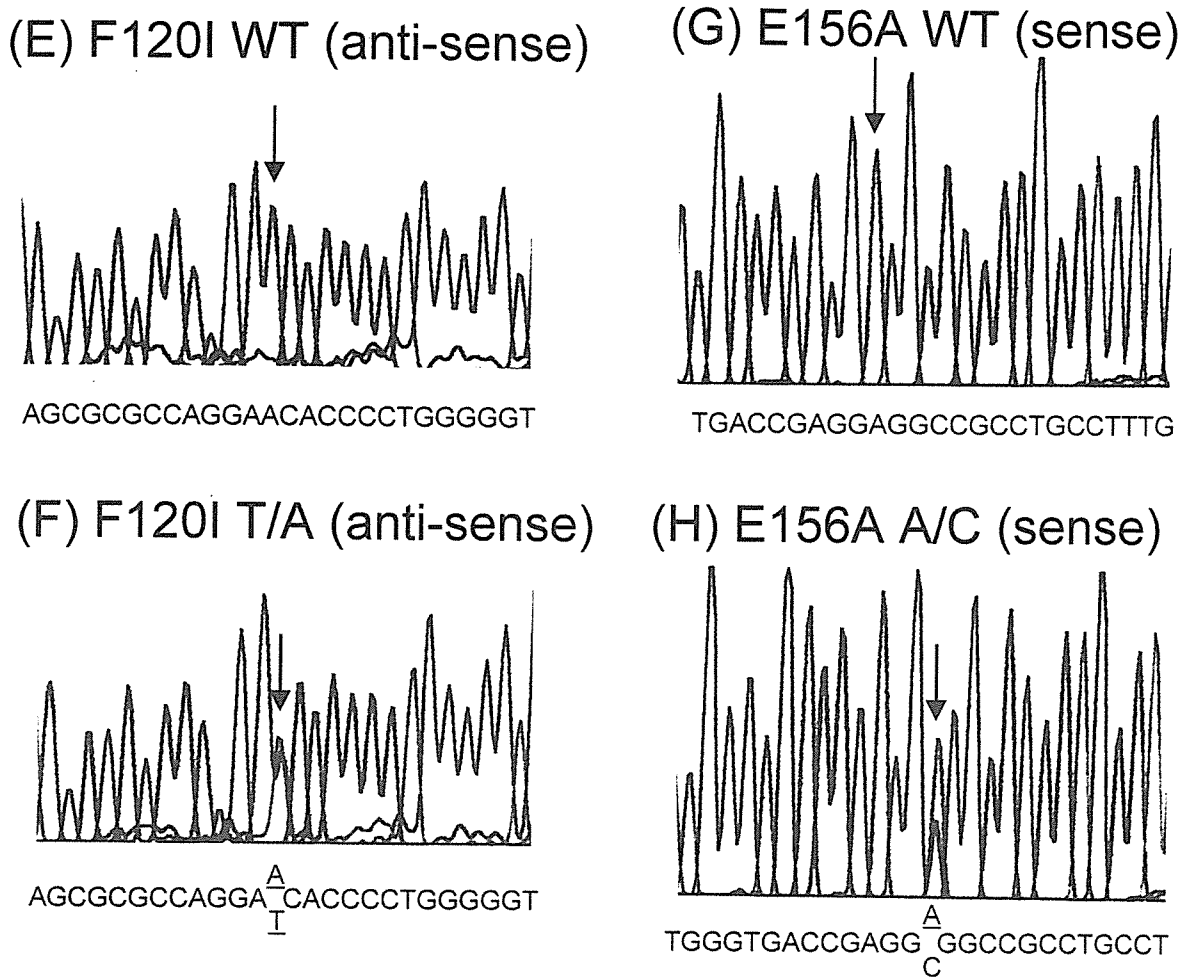


Fig. 1.

19 subjects possessed heterozygous SNPs. This suggested that the 4 subjects possessed aberrant *5 (probably two *CYP2D6* copies), though they were initially judged to have the *CYP2D6**5 haplotype. Thus, we also excluded these 4 subjects from the calculation of SNP frequencies (Table 2). Recently, Ishiguro *et al.* have reported similar observation.¹¹⁾

Among the 5 nonsynonymous SNPs (Table 2), the 1611T>A (F120I, MPJ6_2D6074) was heterozygously detected in an individual who was a homozygous *CYP2D6**10 subject. The 1720A>C (E156A, MPJ6_2D6075) was heterozygously detected in a homozygous *1 individual. The 972C>T, (A90V, MPJ6_2D6073) was found in a subject with *1/*10 genotype. Cloning and sequencing of DNA fragments obtained from the 972C>T subject revealed that 100C and 972T located on the same DNA strand, indicating the association of the MPJ6_2D6073 with the *1 haplotype. Allele-specific PCR analysis revealed that 3172A>C (E334A, MPJ6_2D6082), which was found in a subject with *1/*2 genotype, was associated with

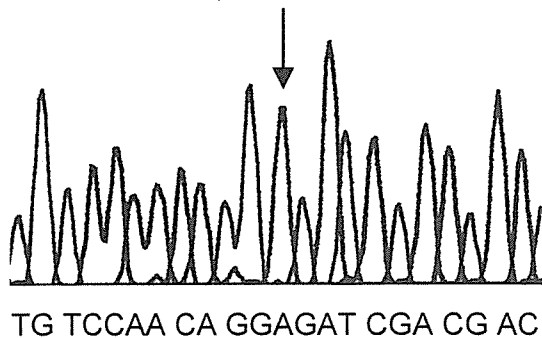
*CYP2D6**2 haplotype. Similarly, by allele-specific PCR analysis, 73C>T (R25W, MPJ6_2D6065) was shown to be linked with C100T, a key SNP of *10 allele. For all the nonsynonymous SNPs, electropherograms are shown for each SNP and the SNP and surrounding nucleotide sequences are written in Fig. 1 (A–J).

Functional characterization of these SNPs is very important especially for those which resulted in the changes of their electronic charges (e.g. E156A and E334A), or for those which are associated with the *10 haplotype (e.g. R25W and F120I).

The known *CYP2D6* alleles, so far observed specifically in Japanese and/or Chinese populations, *14 (1758G>A, G169R), *18 (9 bp insertion, insertion of VPT in exon 9), *21 (2573 ins C, frameshift), and *44 (IVS6+1 G>C, aberrant splicing) were also found, but their frequencies were very low (3/426, 1/426, 3/426 1/426), respectively.

In conclusion, we detected 70 sequence variations in the *CYP2D6* gene including 5 novel nonsynonymous SNPs. These amino acid alterations should further be

(I) E334A WT (sense)



(J) E334A A/C (sense)

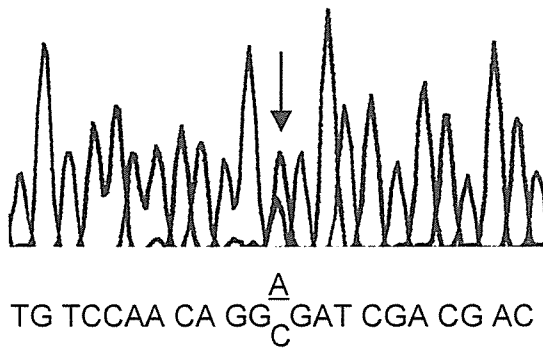


Fig. 1. Electropherogram of 5 nonsynonymous SNPs and their flanking sequences.

The electropherograms are shown in forward or reverse orientation as indicated. Arrows indicate the polymorphic and heterozygous positions and the heterozygous nucleotides are underlined. Bases colored with green, blue, black, and red represent adenine, cytosine, guanine, and thymine, respectively.

evaluated for the influence on catalytic activity, as low activity haplotypes of *CYP2D6* have not been fully elucidated.

Note Added in Proof

The 5 *CYP2D6* haplotypes that possess each of 73C>T (R25W), 972C>T(A90V), 1661T>A (F120I), 1720C>A (E156A), and 3172A>C (E334A) were assigned as *47, *48, *49, *50, and *51, respectively, by the CYP Allele Nomenclature Committee (<http://www.imm.ki.se/CYPalleles>).

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SNP Communication

Seven Novel Single Nucleotide Polymorphisms in the Human SLC22A1 Gene Encoding Organic Cation Transporter 1 (OCT1)

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Full text of this paper is available at <http://www.jssx.org>

Summary: Twenty genetic variations, including seven novel ones, were found in the human *SLC22A1* gene, which encodes organic cation transporter 1, from 116 Japanese individuals. The novel variations were as follows: –94C>A in the 5′-untranslated region (A of the translation start codon is numbered +1 in the cDNA sequence; MPJ6_OC1001), 350C>T (MPJ6_OC1004), IVS1–35T>C (MPJ6_OC1006), 561G>A (MPJ6_OC1010), IVS6+75C>G (MPJ6_OC1014), IVS8+108A>G (MPJ6_OC1017), and 1671_1673delATG (MPJ6_OC1020). The frequencies were 0.082 for IVS1–35T>C, 0.022 for IVS6+75C>G, 0.009 for 561G>A, and 0.004 for the other 4 variations. Among them, 350C>T resulted in the amino acid substitution Pro117Leu, which is located in the large extracellular loop between transmembrane domains 1 and 2. Also, we detected the four previously reported nonsynonymous variations, 123C>G (Phe41Leu), 480C>G (Phe160Leu), 1022C>T (Pro341Leu), and 1222A>G (Met408Val) with frequencies of 0.004, 0.086, 0.168, and 0.810, respectively.

Key words: *SLC22A1* (OCT1); nonsynonymous alteration; intron; novel SNP

Introduction

Human polyspecific organic cation transporter 1 (OCT1), encoded by *SLC22A1*, belongs to the largest superfamily of transporters, the solute carrier family.¹⁾ This transporter is predicted to have 12 transmembrane domains and translocates a wide variety of organic cations including drugs, toxins, and neurotransmitters in an electrochemical potential-dependent manner.^{1,2)}

Since the OCT1 is primarily expressed in the liver (probably in the sinusoidal (basolateral) membrane of hepatocytes), it is expected to play a fundamental role in the uptake of substrates into the liver.^{1–4)} Indeed, *SLC22A1*-knockout mice exhibited decreased liver accumulation of an anti-tumor drug metaiodobenzylguanidine and an antidiabetic drug metformin.^{5,6)}

SLC22A1, located on chromosome 6q26, is adjacent to *SLC22A2* (encoding OCT2) and *SLC22A3* (encoding OCT3), and consists of 11 exons spanning approximately 37 kb.⁷⁾ Several genetic polymorphisms have been found in *SLC22A1* in different ethnic groups. From Caucasians, Kerb *et al.* found 25 polymorphisms, including 8 nonsynonymous ones, three of which showed reduced activities.⁸⁾ Shu *et al.* detected 15 nonsynonymous polymorphisms from 5 different ethnic groups and assessed their functional effects on 1-methyl-

On April 12th, 2004, these variations were not found in the Japanese Single Nucleotide Polymorphisms (JSNP) (<http://snp.ims.u-tokyo.ac.jp/>), dbSNP in the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/SNP/>), or PharmGKB (<http://www.pharmgkb.org/do/>) databases.

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Table 1. Primer sequences used for the analysis of human *SLC22A1*.

	Amplified and sequenced region	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplified length (bp)
1st PCR	Exons 1 to 2	TGACCATTGGAGTTTAAACG	ACCCTCCCCACACTCTTTTA	11,721
	Exons 3 to 8	CTGGCAGAAGTTCCTATGTT	TGGTTGTGTGAAGGTACTTA	12,919
	Exons 9 to 11	GTCTTGATCTCTGACCTCG	ACATCCTCCCTTATGCTTA	6,652
2nd PCR	Exon 1	CTGAACTTCAATTCTCTTCG	CCCACGAACTGCACAATAAA	808
	Exon 2	CCAGGGATACCGAGTTTGAT	ATGCAGCTTGGACTCTGAAA	440
	Exon 3	GCTCAGACTCCTCTTCAGAC	AAAGAGAGGAGGCCATTCTA	531
	Exon 4	AAGGAGAAATGGGAGACACA	GCGTTATGCATGTGGACACC	493
	Exon 5	CACAGAAGGAAGGCTACATA	GGGAGAGCATCAGCCACACT	573
	Exon 6	AGTTGCCTCCTGCTGATTTA	GGTGATCTCCCAAAGGTAAC	569
	Exon 7	GCTCTAGGGCATTCTAAAACC	GGCCCCCAATTTAAAATCT	489
	Exon 8	CCCCTGTTCAATGGAGTCTT	GCTATAATTATGGGTTGACC	521
	Exon 9	TCACTCTAGCCTGTTACCT	CCTAGGAATGGATTCTTATC	539
	Exon 10	TGTACCCCAACAACAAATCC	CAGTAGCTATCATGGGTTTT	575
	Exon 11	AAAAACAGGCTATAAGCTCG	TCTACTGATCTTCAAACGCC	555
Sequencing	Exon 1	AATTCTCTTCGGGCTTAGAC	CTGCACAATAAACATAGCCC	
	Exon 2	GGATACCGAGTTTGATGAAC	TTGGACTCTGAAACACACCT	
	Exon 3	TGGCACTGCTGCTCTGATA	CTGCCCTAGTCACATAAATA	
	Exon 4	GACACACAAGAGAGAAGCCT	CTTTGGAAGACGGCCTGTAG	
	Exon 5	GGCTCCCTTTTGGTCTATAA	TGCTTCACACCCATGACAAG	
	Exon 6	TAGGAAGGGGTATCTCACAT	AAGGGAGAAACAAAAAGAGC	
	Exon 7	TCTCTGACTCATGCCTTTGA	CCTCATCTTTGTTCTCATT	
	Exon 8	ATATGTCATCGTCAACTCCC	AATGCCACTCAATGTCCAAA	
	Exon 9	GCCTGTTACCTCCTCTCAAT	TGAAGCAAGACAGAATAAGC	
	Exon 10	CATTCCCCAGTTATCCTAT	ATAGCAGTTCTGGGAGTAAT	
	Exon 11	CAGGCTGTAGTTTGCTATGC	TAAGTACCCGATACCAATAG	

4-phenylpyridinium uptake.⁹ Five variant proteins showed reduced or diminished activities. As for the Japanese population, four nonsynonymous variations (Phe160Leu, Pro283Leu, Arg287Gly, and Pro341Leu) were found from 48 subjects.¹⁰ Recently, two independent groups reported significantly reduced (Pro341Leu) and abrogated (Pro283Leu and Arg287Gly) transport activities using tetraethylammonium as a substrate.^{11,12}

In this study, we searched for novel SNPs by sequencing all the exons and the surrounding introns of *SLC22A1* from 116 Japanese individuals. Seven novel variations, including one novel nonsynonymous SNP located in exon 1, were identified.

Materials and Methods

Human genomic DNA samples: All of the 116 patients participating in this study were administered cationic antiarrhythmic drugs at the National Cardiovascular Center. The ethical review boards of the National Cardiovascular Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all participating subjects. Genomic DNA extracted from blood leukocytes was used as template in the polymerase chain reaction (PCR).

PCR conditions for DNA sequencing: The genomic and cDNA sequences of *SLC22A1* obtained from GenBank (NT_007422.12 and NM_003057.2, respec-

tively) were used as the reference sequences in this study. First, the entire *SLC22A1* gene was divided into three regions (from exon 1 to 2, from exon 3 to 8, and from exon 9 to 11), and each region was amplified from 50 ng of genomic DNA using 1.25 units of *Z-Taq* (Takara Shuzo, Tokyo, Japan) with 0.2 μ M primers listed in **Table 1** (1st PCR). The first PCR conditions were 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec. Next, each exon was amplified by *Ex-Taq* (0.625 units) (Takara Shuzo) with an appropriate set of *SLC22A1*-specific primers (0.2 μ M) designed in the introns, as listed in **Table 1** (2nd PCR). The second round PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 2 min, and then a final extension for 7 min at 72°C. The PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in **Table 1** (Sequencing). The excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). All the novel SNPs were confirmed by repeated sequencing of amplified products from the 1st PCR.